#12/Declaration

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of:

KILGER, Christian; PÄÄBO Svante

Serial Number: 08/991,184 Group Art Unit: 1634

Filed: December 16, 1997 Examiner: Horlick, K.

For: METHOD FOR UNCOUPLED, DIRECT, EXPONENTIAL AMPLIFICATION AND SEQUENCING OF DNA MOLECULES WITH THE ADDITION OF A SECOND THERMOSTABLE DNA POLYMERASE AND ITS APPLICATION

SECOND DECLARATION UNDER 37 CFR 1.132

Assistant Commissioner for Patents
U.S. Patent and Trademark Office
Washington, D.C. 20231

Sir:

I, Christian Kilger, hereby declare and state:

1. I have a doctoral degree in natural science which was conferred upon me by the Faculty of Biology, the Ludwig-Maximilians-Universität at Munich, Germany, in 1996.

2. I am a co-inventor of the present application and am knowledgeable of the art to which the present invention pertains.

3. Following the disclosure in Köster et al (US 5,928,906, hereinafter referred to as US '906), one skilled in the art would have to perform unreasonable experimentation in order to practice the method of US '906 for simultaneous amplification and sequencing of DNA molecules.

4. The method taught by the only working example, i.e. Example 1, of US '906 was inoperative as shown by the experiments described below.

5. The following experiments (I)-(IV) were conducted under my supervision or direction.

Experiment (I): Ratio of Taq DNA polymerase to AmpliTaqFS polymerase = 1 : 1.6 up to 1 : 32

A plasmid DNA, pQE30 containing a 800 bp insert, was subjected to a simultaneous-amplification-and-sequencing method essentially identical to the method disclosed in Example 1 of US '906.

A-reaction for a ratio of 1 : 1.6: A 4 µl solution containing 8 µM ddATP, 80 µM each of dATP, dCTP, dGTP, and dTTP, 500 mM Tris-HCl (pH 9), 25 mM $MgCl_2$, 1 pmol forward labeled primer, and thermostable pyrophosphatase was added to 50 ng of the plasmid DNA (1µl), 1 U of Taq DNA polymerase (1µl), 1.6 U of AmpliTaqFS polymerase, and 10 pmol reverse primer (0.5µl).

C-reaction for a ratio of 1 : 1.6: A 4 µl solution containing 8 µM ddCTP, 80 µM each of dATP, dCTP, dGTP, and dTTP, 500 mM Tris-HCl (pH 9), 25 mM $MgCl_2$, 1 pmol forward labeled primer, and thermostable pyrophosphatase was added to 50 ng of the plasmid DNA (1µl), 1 U of Taq DNA polymerase (1µl), 1.6 U of AmpliTaqFS polymerase, and 10 pmol reverse primer (0.5µl).

G-reaction for a ratio of 1 : 1.6: A 8 µl solution containing 8 µM ddGTP, 80 µM each of dATP, dCTP, dGTP, and dTTP, 500 mM Tris-HCl (pH 9), 25 mM $MgCl_2$, 1 pmol forward labeled primer, and thermostable pyrophosphatase was added to 50 ng of the plasmid DNA (1µl), 1 U of Taq DNA polymerase (1µl), 1.6 U of AmpliTaqFS polymerase, and 10 pmol reverse primer (0.5µl).

T-reaction for a ratio of 1 : 1.6: A 8 µl solution containing 8 µM ddTTP, 80 µM each of dATP, dCTP, dGTP, and dTTP, 500 mM Tris-HCl (pH 9), 25 mM $MgCl_2$, 1 pmol forward labeled primer, and thermostable was added to 50 ng of the plasmid DNA (1µl), 1 U of Taq DNA polymerase (1µl), 1.6 U of AmpliTaqFS polymerase, and 10 pmol reverse primer (0.5µl).

The reaction mixtures were incubated under the following cycling conditions: an initial denaturation step at 95°C for 4 min, followed by 15 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 60 seconds at 72°C. The amplification and sequencing reaction was completed by 15 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 60 seconds at 72°C.

Sequencing gels were run using the reaction mixtures and a picture of the sequencing gel is shown in Figure 1 attached. The lanes labeled as 2, 3, 4 ,5 and 6 in Fig. 1 represent the sequencing gels of the A-, C-, G- and T-reactions of Experiment (I) as described above.

Reaction conditions "essentially identical" to US '906 means that there are only minor differences which are mainly due to the non-availability of certain materials such as plasmids and target sequences which were used in the Example of US'906. The differences listed below would not change the results obtained.

1) The DNA sequence of the vector used (pQE30 instead of p0M89, the insert (800 bp instead of 400 bp) and therefore also the primers are different.
2) The label used in the present experiment to detect the sequence ladder are IRD-labels instead of JOE/FAM/TAMRA or ROX labeled primers.

3) dGTP was used instead of 7-deaza-dGTP.

Discussion of experiment I:

A plasmid DNA, pQE30 containing a 800 bp insert, was subjected to a simultaneous-amplification-and-sequencing method essentially identical to the method disclosed in Example 1 of US '906. The procedure described in Experiment (I) above was followed using a ratio of Taq DNA polymerase to AmpliTaqFS polymerase = 1 : 1.6 up to 1 : 32.

No detectable sequence ladder in the A-, C-, G- and T-reactions was obtained when the ratio of Taq DNA polymerase to AmpliTaqFS polymerase = 1 : 1.6 up to 1 : 32 was used in a process similar to that of Example 1 of US '906.

Experiment (II): Ratio of Taq DNA polymerase to ThermoSequenase polymerase = 1 : 1.6 up to 1 : 32.

A plasmid DNA, pQE30 containing a 800 bp insert, was subjected to a simultaneous-amplification-and-sequencing method essentially identical to the method disclosed in Example 1 of US '906. The procedure described in Experiment (I) above was followed except that ThermoSequenase polymerase, instead of AmpliTaqFS polymerase, was used.

No detectable sequence ladder in the A-, C-, G- and T-reactions was obtained when the ratio of Taq DNA polymerase to ThermoSequenase polymerase 1 : 1.6 up to 1 : 32 was used in a process essentially identical to that of Example 1 of US '906.

The lanes labeled as 7, 8, 9 ,10 and 11 in Fig. 1 represent the sequencing results of the A-, C-, G- and T-reactions of Experiment (II) as described above.

Experiment (III): DEXTAQ conditions, Ratio of Taq DNA polymerase to AmpliTaqFS polymerase = 1 : 1.6 up to 1 : 32.

A plasmid DNA, pQE30 containing a 800 bp insert, was subjected to a simultaneous-amplification-and-sequencing method essentially identical to the method disclosed in the present application (DEXTAQ).

DEXTAQ conditions:

A-reaction (also C, G, T- reaction):

5 µl volume containing:

12.5 ng template pQE 30 with 800 bp insert, 0.25 pmol labeled forward primer, 0.1 U thermostable pyrophosphatase, 0.25 U Taq DNA polymerase, 2.5 pmol reverse primer, 100 mM Tris (pH8), 5 mM $MgCl_2$, 1.6 U up to 32 U of AmpliTaq FS or Thermosequenase (in case of Thermosequenase 20 mM Tris (pH 9.5) and 4.25 mM $MgCl_2$ were used)

additional 2 µl volume containing:

5 µM ddATP (in case of the C-reaction 5 µM ddCTP, in case of the G-reaction 5 µM ddGTP, in case of the T-reaction 5 µM ddTTP) and 1 mM each dNTP (total volume 7 µl).

The reaction mixtures were incubated under the following cycling conditions: an initial denaturation step at 95°C for 4 min, followed by 15 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 60 seconds at 72°C. The amplification and sequencing

reaction was completed by 15 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 60 seconds at 72°C.

A clear detectable sequence ladder in the A-, C-, G- and T-reactions was obtained .

The lanes labeled as 13, 14, 15 ,16 and 17 in Fig. 1 represent the sequencing results of the A-, C-, G- and T-reactions of Experiment (III) as described above.

Experiment (IV): DEXTAQ conditions, Ratio of Taq DNA polymerase to ThermoSequenase polymerase = 1 : 1.6 up to 1 : 32.

A plasmid DNA, pQE30 containing a 800 bp insert, was subjected to a simultaneous-amplification-and-sequencing method essentially identical to the method disclosed in the present application (DEXTAQ) and as described in Experiment (III) except that ThermoSequenase polymerase, instead of AmpliTaqFS polymerase, was used.

A clear detectable sequence ladder in the A-, C-, G- and T-reactions was obtained .

The lanes labeled as 18, 19, 20 ,21 and 22 in Fig. 1 represent the sequencing gels of the A-, C-, G- and T-reactions of Experiment (IV) as described above.

Detailed description of Fig.1.

lane 1 and 12: standard cycle sequencing reactions as control reactions for gel conditions: (5µl volume containing: 1µg plasmid DNA, 2 pmol each Primer, 10 U AmpliTaq FS, 100 mM Tris (pH 8), 2.5 mM

$MgCl_2$), additional 2 µl volume containing : 2.5 mM one ddNTP and 750 µM each dNTP)

cycled at:[(97°C – 15 sec, 55°C - 30 sec, 68°C – 30 sec) x 30]

lane 2 to 6: conditions essentially identical to US ´906 (Sequenom patent) with a ratio Taq : AmpliTaq FS varying from 1 : 1.6 (lane 2), 1:4 (lane 3), 1:8 (lane 4), 1:16 (lane 5) up to 1:32 (lane 6)

lane 7 to 11: conditions essentially identical to US ´906 (Sequenom patent) with a ratio Taq : Thermosequenase starting 1 : 1.6 (lane 7) up to 1:16 (lane 10), 1 : 32 (lane 11)

lane 13 to 17: conditions essentially identical to the DEXTAQ application with a ratio Taq : AmpliTaq FS starting from 1 : 1.6 (lane 13) up to 1:16 (lane 16), 1 : 32 (lane 17)

lane 18 to 22: conditions essentially identical to the DEXTAQ application with a ratio Taq : Thermosequenase starting from 1 : 1.6 (lane 18) up to 1:16 (lane 21), 1 : 32 (lane 22)

6. The results of Experiments (I)-(IV) demonstrate that the disclosure of US '906 fails to adequately teach one skilled in the art how to perform the method of

simultaneous amplification and sequencing of DNA. Experiment (I) shows that performing a procedure essentially identical to the method disclosed in the only working example, Example 1, of US '906 failed to simultaneously amplify and sequence a plasmid DNA using the same two DNA polymerases, Taq DNA polymerase and AmpliTaqFS polymerase, in the same ratio of 1 : 1.6 as disclosed in Example 1 of US '906. Despite the fact that US '906 discloses that Taq DNA polymerase and ThermoSequenase polymerase can be used in the method of US '906 (column 7, lines 56, 59 and 60), Experiment (II) shows that using the reagent concentrations and reaction conditions essentially identical to those disclosed in Example 1 of US '906 failed to result in simultaneous amplification and sequencing of the plasmid DNA when Taq DNA polymerase and ThermoSequenase polymerase were employed as the two DNA polymerases at the ratio of 1 : 1.6 taught by the only working example of US '906. Even when the reagent concentrations and reaction conditions similar to those disclosed in Example 1 of US '906 were used with Taq DNA polymerase and AmpliTaqFS polymerase or Thermosequenase, respectively, at a ratio of up to 1 : 32, which was much higher than the ratio of 1 : 1.6 taught by Example 1 of US '906 the method did not yield a detectable sequence ladder with the A-, C-, G- and T-reactions (see Experiments I and II). However, Experiments (III) and (IV) show that when the reagent concentrations and reaction conditions taught by the present application were used a simultaneous amplification and sequencing of the plasmid DNA could be achieved with any enzyme ratio using either AmpliTaqFS polymerase or Thermosequenase.

With **the failures** of the method taught by the only working example of US '906 the guidance provided by the disclosure of US '906 is thus grossly inadequate. It would require undue experimentation for one skilled in the art to practice the method disclosed in US '906.

7. For the sake of completeness and in order to fully fulfill my duty of disclosure I would like to bring to the Examiners attentions experiments conducted under similar but different conditions as taught by the only working example, i.e. Example 1, of US'906. The main difference is the amount of plasmid DNA which was subjected to the sequencing method similar to the method disclosed in Example 1 of US'906. In these experiments 100 ng of the plasmid DNA were subjected to amplification and sequencing. Further a puc18 containing a 1500 bp insert was used.

8. The following experiments (V)-(VIII) were conducted under my supervision or direction.

(V) Ratio of Taq DNA polymerase to AmplifTaqFS polymerase = 1 : 1.6

A plasmid DNA, pUC18 containing a 1500 bp insert, was subjected to a simultaneous-amplification-and-sequencing method similar to the method disclosed in Example 1 of US '906.

A-reaction: A 4 µl solution containing 8 µM ddATP, 80 µM each of dATP, dCTP, dGTP, and dTTP, 500 mM Tris-HCl (pH 9), 25 mM $MgCl_2$, 1 pmol forward primer, and thermostable pyrophosphate was added to 100 ng of the plasmid DNA (1µl), 1 U of Taq DNA polymerase (1µl), 1.6 U of AmpliTaqFS polymerase, and 10 pmol reverse primer (0.5µl).

C-reaction: A 4 µl solution containing 8 µM ddCTP, 80 µM each of dATP, dCTP, dGTP, and dTTP, 500 mM Tris-HCl (pH 9), 25 mM $MgCl_2$, 1 pmol forward primer, and thermostable pyrophosphate was added to 100 ng of the plasmid DNA (1µl), 1 U of Taq DNA polymerase (1µl), 1.6 U of AmpliTaqFS polymerase, and 10 pmol reverse primer (0.5µl).

G-reaction: A 8 µl solution containing 8 µM ddGTP, 80 µM each of dATP, dCTP, dGTP, and dTTP, 500 mM Tris-HCl (pH 9), 25 mM $MgCl_2$, 1 pmol forward primer, and thermostable pyrophosphate was added to 100 ng of the plasmid DNA (1µl), 1 U of Taq DNA polymerase (1µl), 1.6 U of AmpliTaqFS polymerase, and 10 pmol reverse primer (0.5µl).

T-reaction: A 8 µl solution containing 8 µM ddTTP, 80 µM each of dATP, dCTP, dGTP, and dTTP, 500 mM Tris-HCl (pH 9), 25 mM $MgCl_2$, 1 pmol forward primer, and thermostable pyrophosphate was added to 100 ng of the plasmid DNA (1µl), 1 U of Taq DNA polymerase (1µl), 1.6 U of AmpliTaqFS polymerase, and 10 pmol reverse primer (0.5µl).

The reaction mixtures were incubated under the following cycling condition: an initial denaturation step at 95°C for 4 min, followed by 15 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 60 seconds at 72°C. The amplification and sequencing reaction was completed by 15 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 60 seconds at 72°C.

Sequencing gels were obtained from the reaction mixtures and a picture of the sequencing gels is shown in Figure 2 attached. The lanes labeled as 2, 3, 4 and 5 in

Fig. 2 represent the sequencing gels of the A-, C-, G- and T-reactions of Experiment (V).

Lane 1: Control sequencing gels which demonstrate that a sequence ladder was detected, standard cycle sequencing reactions as control reactions for gel conditions (5µl volume containing: 1µg plasmid DNA, 2 pmol each Primer, 10 U AmpliTaq FS, 100 mM Tris (pH 8), 2.5 mM $MgCl_2$), additional 2 µl volume containing : 2.5 mM one ddNTP and 750 µM each dNTP)

cycled at:[(97°C – 15 sec, 55°C - 30 sec, 68°C – 30 sec) x 30]

Lanes 2-5: The sequencing gels of the A-, C-, G- and T-reactions, respectively, of Experiment (I). No sequence ladder was detected in lanes 2-5 when the ratio of Taq DNA polymerase to AmplifTaqFS polymerase = 1 : 1.6 was used in a process similar to that of Example 1 of US '906.

(VI) Ratio of Taq DNA polymerase to AmplifTaqFS polymerase = 1 : 16

A plasmid DNA, pUC18 containing a 1500 bp insert, was subjected to a simultaneous-amplification-and-sequencing method similar to the method disclosed in Example 1 of US '906, except that 16 U, instead of 1.6 U, of AmpliTaqFS polymerase was used.

The sequencing gels of the A-, C-, G- and T-reactions were shown in Lanes 6-9 of Figure 2 attached. Lanes 6-9 demonstrate that a detectable sequence ladder was obtained when the ratio of Taq DNA polymerase to AmplifTaqFS polymerase = 1 : 16 was used in a process similar to that of Example 1 of US '906.

(VII) Ratio of Taq DNA polymerase to ThermoSequenase polymerase = 1 : 1.6

A plasmid DNA, pUC18 containing a 1500 bp insert, was subjected to a simultaneous-amplification-and-sequencing method similar to the method disclosed in Example 1 of US '906. The procedure described in Experiment (V) above was followed except that 1.6 U of ThermoSequenase polymerase, instead of 1.6 U of AmpliTaqFS polymerase, was used.

No detectable sequence ladder in the A-, C-, G- and T-reactions was obtained when the ratio of Taq DNA polymerase to ThermoSequenase polymerase = 1 : 1.6 was used in a process similar to that of Example 1 of US '906.

(VIII) Ratio of Taq DNA polymerase to ThermoSequenase polymerase = 1 : 16

A plasmid DNA, pUC18 containing a 1500 bp insert, was subjected to a simultaneous-amplification-and-sequencing method similar to the method disclosed in Example 1 of US '906. The procedure described in Experiment (V) above was followed except that 16 U of ThermoSequenase polymerase, instead of 1.6 U of AmpliTaqFS polymerase, was used.

No detectable sequence ladder in the A-, C-, G- and T-reactions was obtained when the ratio of Taq DNA polymerase to ThermoSequenase polymerase = 1 : 16 was used in a process similar to that of Example 1 of US '906.

9. The results of Experiments (V)-(VIII) demonstrate that the disclosure of US '906 fails to adequately teach one skilled in the art how to perform the method of simultaneous amplification and sequencing of DNA. Experiment (V) shows that

performing a procedure similar to the method disclosed in the only working example, Example 1, of US '906 failed to simultaneously amplify and sequence a plasmid DNA using the same two DNA polymerases, Taq DNA polymerase and AmplifTaqFS polymerase, in the same ratio of 1 : 1.6 as disclosed in Example 1 of US '906. Despite the fact that US '906 discloses that Taq DNA polymerase and ThermoSequenase polymerase can be used in the method of US '906 (column 7, lines 56, 59 and 60), Experiment (VII) show that using the reagent concentrations and reaction conditions similar to those disclosed in Example 1 of US '906 failed to result in simultaneous amplification and sequencing of the plasmid DNA when Taq DNA polymerase and ThermoSequenase polymerase were employed as the two DNA polymerases at the ratio of 1 : 1.6 taught by the only working example of US '906. Only when the reagent concentrations and reaction conditions similar to those disclosed in Example 1 of US '906 were used with Taq DNA polymerase and AmpliTaqFS polymerase at a ratio of 1 : 16, which was much higher than the ratio of 1 : 1.6 taught by Example 1 of US '906 did the method yield a detectable sequence ladder with the A-, C-, G- and T-reactions. However, Experiment (VIII) shows that when the reagent concentrations and reaction conditions similar to those disclosed in Example 1 of US '906 were used with Taq DNA polymerase and ThermoSequenase polymerase at a ratio of 1 : 16, no simultaneous amplification and sequencing of the plasmid DNA could be achieved.

With **two failures** of the method taught by the only working example of US '906 using two polymerases at a ratio of 1 : 1.6 and with the fact that using the reagent concentrations and reaction conditions similar to those disclosed in the only working example of US '906 was successful in the simultaneous amplification and sequencing of

DNA only when Taq DNA polymerase and AmpliTaqFS polymerase were used at a ratio **10 times** that taught by the only working example of US '906 (but not successful when Taq DNA polymerase and ThermoSequenase polymerase were used at a ratio of 1 : 16), the guidance provided by the disclosure of US '906 is thus grossly inadequate. Further, one has to bear in mind that the amount of plasmid DNA used in experiments (V) – (VIII) was two-fold the amount Sequenom's Example 1 shows. Using the two-fold amount of target DNA facilitates the sequencing procedure considerably and is therefore misleading when evaluating the operativeness of a method. If a high amount of target DNA is available no amplification at all would be needed and prior art cycle sequencing could result in a readable sequencing ladder (cycle sequencing uses one polymerases which discriminates against ddNTP's). Therefore, results obtained by Experiments (I) – (IV) are regarded to be more relevant in order to evaluate the operativeness of a method according to US'906 because essentially identical experimental conditions are applied. Thus, it would require undue experimentation for one skilled in the art to practice the method disclosed in US '906.

10. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine and/or imprisonment under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-identified patent application or any patent issuing therefrom.

Date: 28.2.2000
[Date Declaration Signed]

[Declarant's Signature]

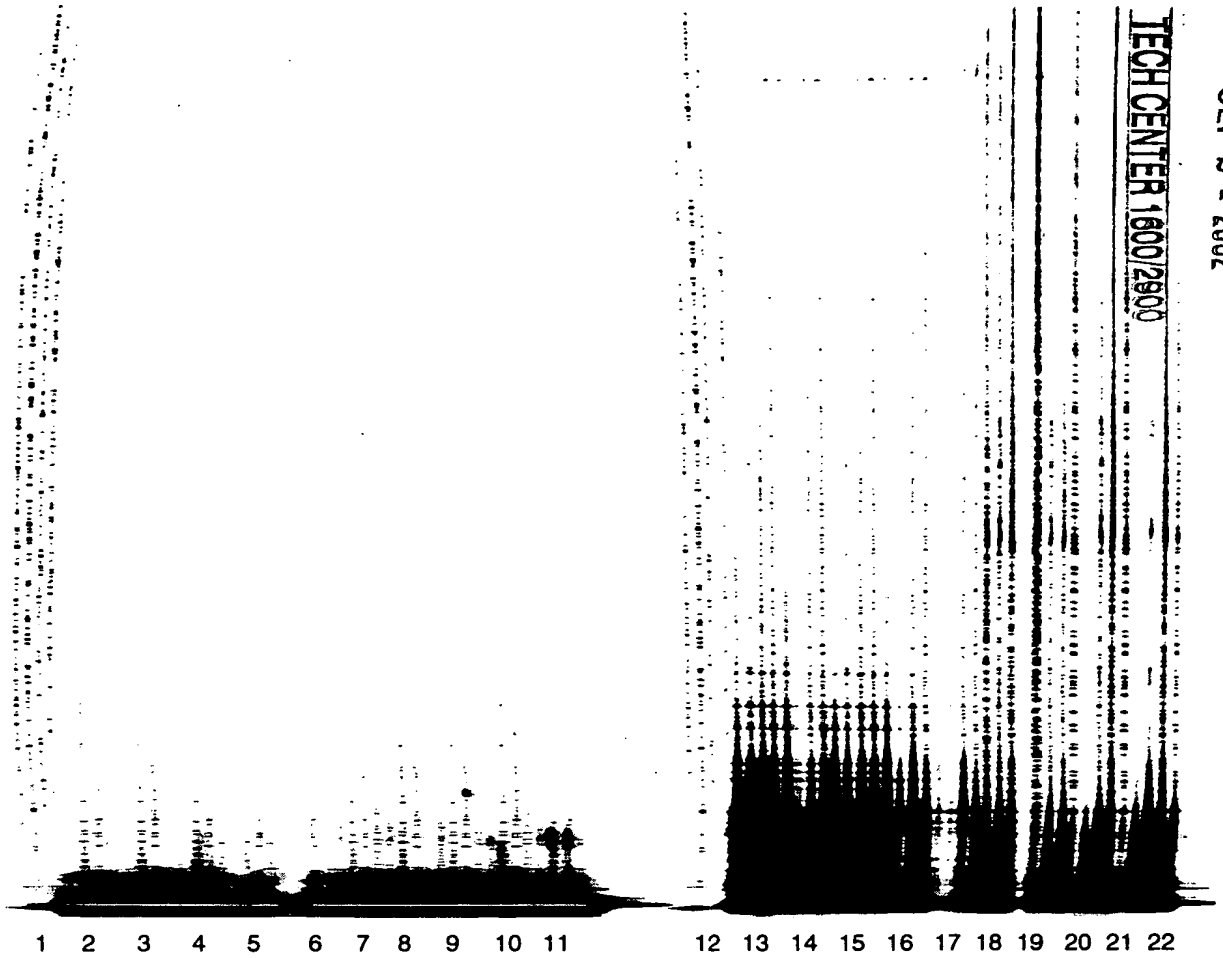

FIGURE 1

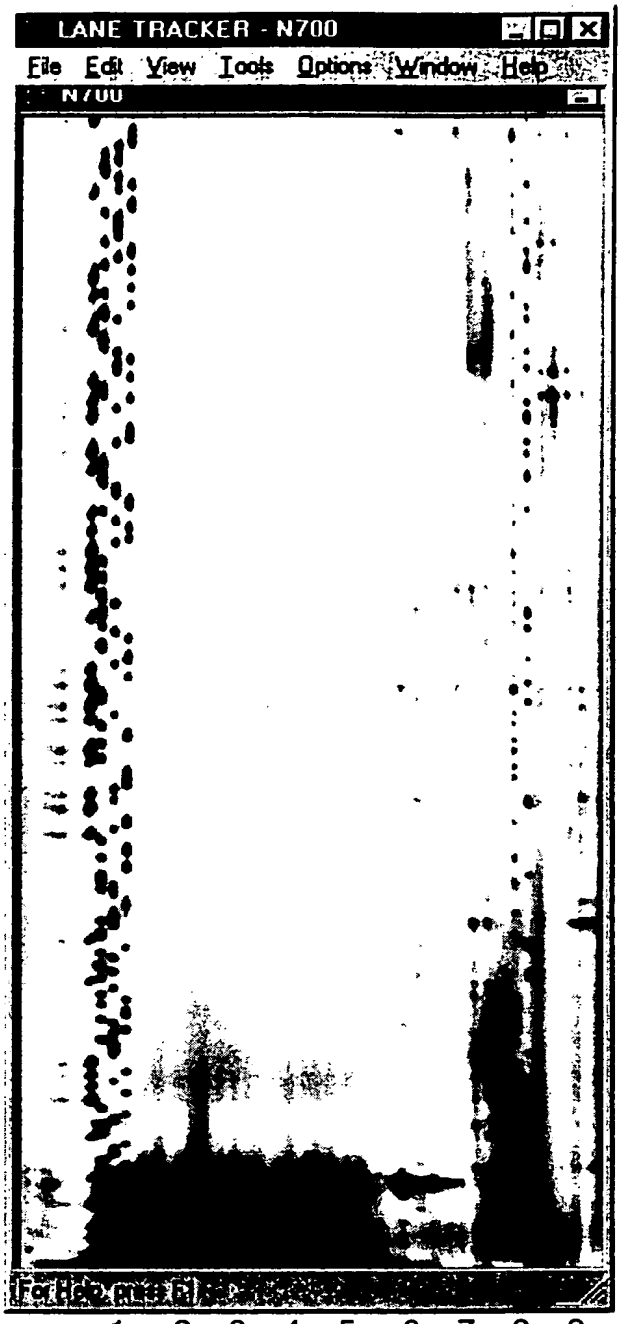

FIGURE 2